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Advances in Brief

Essential Role of Tumor Necrosis Factor α (TNF-α) in Tumor Promotion as Revealed by TNF-α-deficient Mice[1]

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Abstract

To examine the hypothesis that tumor necrosis factor (TNF) α is an essential cytokine in carcinogenesis, we conducted two-stage carcinogenesis experiments with an initiator, 7,12-dimethylbenz(*a*)anthracene (DMBA), plus either of two tumor promoters, okadaic acid and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), on the skin of TNF-α-deficient ($TNF^{-/-}$) mice. $TNF^{-/-}$ mice treated with DMBA plus okadaic acid developed no tumors for up to 19 weeks, and at 20 weeks, the percentage of tumor-bearing $TNF^{-/-}$ mice was 10%, whereas the percentage of tumor-bearing $TNF^{+/+}$ mice was 100%. In $TNF^{-/-}$ mice treated with DMBA plus TPA, tumor onset was delayed 4 weeks, and the time to development of small tumors in 100% of mice was 9 weeks later than that seen in $TNF^{+/+}$ CD-1 mice. The average number of tumors in TPA-treated $TNF^{-/-}$ mice was 2.8, compared with 11.8 for $TNF^{+/+}$ CD-1 mice. To understand the residual tumor-promoting activity in $TNF^{-/-}$ mice, we also investigated the possible significance of interleukin (IL) 1 as an additional cytokine in tumor promotion. A single application of TPA and okadaic acid increased *IL-1α* and *IL-1β* gene expression in $TNF^{-/-}$ mice. All of our results demonstrate that TNF-α is the key cytokine for tumor promotion in mouse skin and, very possibly, for carcinogenesis in humans as well.

Introduction

The study of tumor promotion is not new but is expected to be increasingly applicable in human carcinogenesis. A large number of two-stage carcinogenesis experiments with chemical tumor promoters in various target organs have been conducted in the last decade (1) in search of the essential and common molecule that causes tumor development in humans. Our studies with tumor promoters of the okadaic acid class have provided strong evidence that TNF-α[3] is the central mediator of tumor promotion and, furthermore, that TNF-α released from initiated cells or various neighboring cells induces clonal growth in these initiated cells (2, 3). Two mice models are now available to examine our hypothesis *in vivo*: (*a*) TNF-α transgenic mice; and (*b*) TNF-α-deficient ($TNF^{-/-}$) mice. We first examined TNF-α transgenic mice, and we found that prolonged overexpression of TNF-α in the lungs induced interstitial pneumonitis (4). The results encouraged us to conduct a two-stage carcinogenesis experiment in TNF-α transgenic mice with a proper initiator. On the other hand, we thought that the study of tumor promotion in $TNF^{-/-}$ mice would be more significant in revealing the ultimate role of TNF-α.

We have generated $TNF^{-/-}$ mice by replacement-type homologous recombination using a targeting vector designed to inactivate the *TNF-α* gene (5). Although $TNF^{-/-}$ mice lack both the soluble and membrane forms of TNF-α (5, 6), $TNF^{-/-}$ mice develop normally and have no gross structural or morphological abnormalities. Cytokine production induced by lipopolysaccharide in $TNF^{-/-}$ mice, including the production of IL-1α, IL-6, IL-10, IL-12, and IFN-γ, appears to be essentially intact (5). Based on our assumption that tumor development induced by chemical tumor promoters will be compromised in $TNF^{-/-}$ mice, we conducted a series of two-stage carcinogenesis experiments using the initiator DMBA plus a tumor promoter (either okadaic acid or TPA). We chose okadaic acid and TPA as tumor promoters because of their potent tumor-promoting activities in mouse skin, and also because they have different mechanisms of action (7).

In this study, we present the first evidence that tumor promotion by okadaic acid and TPA is critically dependent on TNF-α. Specifically, okadaic acid did not show any tumor-promoting activity in $TNF^{-/-}$ mice after up to 19 weeks of tumor promotion, whereas okadaic acid induced strong tumor-promoting activity in $TNF^{+/+}$ mice. Tumor development in TPA-treated $TNF^{-/-}$ mice was delayed, and the average number of tumors/mouse, as well as tumor size, was dramatically reduced compared with that of $TNF^{+/+}$ CD-1 mice. Although tumorigenesis in $TNF^{-/-}$ mice was significantly depressed compared with that seen in $TNF^{+/+}$ mice, some tumor development was still observed. We present evidence that IL-1, a cytokine that shares some signal transduction pathways with TNF-α, might contribute to this residual tumor-promoting activity. Thus, whereas our studies in $TNF^{-/-}$ mice suggest a role for IL-1 in tumor promotion, they clearly demonstrate that TNF-α is the essential cytokine in tumor promotion in mouse skin.

Materials and Methods

Mice. $TNF^{-/-}$ and $TNF^{+/+}$ mice on the 129/Svj background were generated by mating heterozygous mice as described previously (5). Male and female 8–10-week old mice were used in this study. The $TNF^{+/+}$ CD-1 mice used as controls were obtained from Charles River Inc. (Kanagawa, Japan).

Chemicals. Okadaic acid was isolated from a black sponge, *Halichondria okadai*, as described previously (8). DMBA and TPA were purchased from Sigma Chemical Co. (St. Louis, MO) and Chemsyn Science Laboratory (Lenexa, KS), respectively. Recombinant human TNF-α, IL-1α, and IL-1β were purchased from Genzyme (Cambridge, MA). BALB/3T3 cells and a v-Ha-*ras*-transfected BALB/3T3 clone (Bhas 42) were provided by the Japanese Cancer Center Research Resources Bank (Tokyo, Japan; Ref. 2).

Two-Stage Carcinogenesis Experiments on Mouse Skin. The skin on the backs of $TNF^{-/-}$ 129/Svj, $TNF^{+/+}$ 129/Svj, and $TNF^{+/+}$ CD-1 mice (8 weeks old) was initiated with a single application of 100 μg of DMBA. From 1 week after initiation, okadaic acid (5.0 μg) or TPA (2.5 μg) dissolved in 100 μl of acetone was applied topically twice a week until week 20, as described previously (8). Each group consisted of 10 mice. The number of tumors more than 1 mm in diameter was counted weekly.

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[3] The abbreviations used are: TNF, tumor necrosis factor; DMBA, 7,12-dimethylbenz(*a*)anthracene; IL, interleukin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NF-κB, nuclear factor κB.

Effects of IL-1α, IL-1β, and TNF-α on the Clonal Growth of v-Ha-*ras*-transfected BALB/3T3 (Bhas 42) Cells. Bhas 42 cells (1×10^4) were seeded on a 96-well plate in MEM containing 10% FCS and then treated with various concentrations of *IL*-1α, *IL*-1β, and TNF-α for 8 days. After treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, the numbers of transformed foci were counted according to the reported criteria (9). Each of the results was the mean ± SE of quadruplicate analyses.

Expression of *IL*-1α, *IL*-1β, and *TNF*-α Genes in Mouse Skin. Okadaic acid or TPA was applied to the skin of the backs of a TNF$^{-/-}$ mouse or a TNF$^{+/+}$ CD-1 mouse 48 h after shaving. Total RNA was isolated from the skin of three mice at various times after application by the acid guanidium/phenol/chloroform extraction method, and polyadenylated RNA was isolated from total RNA using Oligotex-dT30 (Super) (Nippon Roche, Tokyo, Japan). Expression of *IL*-1α, *IL*-1β, and *TNF*-α genes was analyzed by reverse transcription-PCR as follows (10): polyadenylated RNA was reverse-transcribed to cDNA with murine leukemia virus reverse transcriptase; and cDNA was amplified using specific primers for *IL*-1α, *IL*-1β, and *TNF*-α by 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s, in presence of [^{32}P]dCTP. PCR products were separated by 5% PAGE, and radioactivity was counted using a BAS 2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as a control (4). The results were confirmed by two independent experiments.

Statistical Analysis. The Student's *t* test was performed to compare the percentage of tumor-bearing mice, the average number of tumors/mouse, and the stimulation of the clonal growth of Bhas 42 cells. Statistical significance levels were $P \leq 0.05$ and $P \leq 0.01$.

Results and Discussion

Absence of Tumor Formation by Okadaic Acid in DMBA-initiated Skin of TNF$^{-/-}$Mice. The dose of okadaic acid used here (5 μg/application) usually induces tumors in 100% of TNF$^{+/+}$ CD-1 mice by week 20 in a two-stage carcinogenesis experiment (7). However, repeated applications of okadaic acid did not produce any tumors on the skin of TNF$^{-/-}$ mice by week 19, and only one small tumor in one mouse was seen at week 20 (Fig. 1). The same dose of okadaic acid induced the first tumor on the skin of TNF$^{+/+}$ 129/Svj mice during week 11 of tumor promotion, and by week 17, 100% of these mice had developed tumors. Similarly, 100% of TNF$^{+/+}$ CD-1 mice had developed tumors by week 17 of tumor promotion. The average number of tumors/mouse at week 20 was 0.1 for TNF$^{-/-}$ mice, 4.0 for TNF$^{+/+}$ 129/Svj mice, and 8.8 for TNF$^{+/+}$ CD-1 mice (Fig. 1). These results clearly demonstrate that tumor promotion in TNF$^{-/-}$ mice is refractory to the effects of okadaic acid.

Delay in Tumor Formation by TPA in DMBA-initiated Skin of TNF$^{-/-}$ Mice. Next we examined the tumor-promoting activity of TPA in TNF$^{-/-}$ mice. For this experiment, we used 2.5 μg of TPA per application, which usually produces tumors in 100% of TNF$^{+/+}$ CD-1 mice by week 15 (11). In TNF$^{-/-}$ mice treated with DMBA plus TPA, the first tumor developed during week 11, 4 weeks later than in TNF$^{+/+}$ CD-1 mice (Fig. 2). In week 11, the percentage of tumor-bearing mice was only 20% for TNF$^{-/-}$ mice but was 100% for TNF$^{+/+}$ CD-1 mice, and the average number of tumors/mouse was 0.2 for TNF$^{-/-}$ mice and 3.2 for TNF$^{+/+}$ CD-1 mice. After week 11, the tumor incidence in TNF$^{-/-}$ mice gradually increased, reaching 100% at week 20, a 9-week delay. The average number of tumors/mouse in TNF$^{-/-}$ mice was 2.8 at week 20, significantly fewer than the 11.8 tumors/mouse observed in TNF$^{+/+}$ CD-1 mice.[4] In addition, the diameters of tumors in TNF$^{-/-}$ mice were significantly smaller than those in TNF$^{+/+}$ CD-1 mice. These results indicate that TNF-α is the essential cytokine in tumor promotion. However, a small number of tumors did develop in TNF$^{-/-}$ mice treated with DMBA plus TPA, which raised the next question: what other cytokines besides TNF-α are involved in tumor promotion? It is possible that other members of the TNF-α ligand family may be involved. However, there are a number of observations consistent with a role for IL-1 in this process. There are a number of similarities in the signal transduction pathways induced by TNF-α and IL-1 (12). *In vitro*, IL-1 stimulates the proliferation of mouse keratinocytes, whereas *in vivo*, TPA induced *IL-1* gene expression in mouse skin (13, 14). In light of these reports, we examined the tumor-promoting activity of IL-1 *in vitro*.

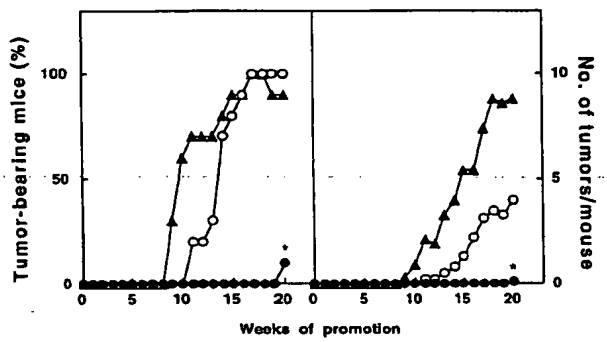

Fig. 1. Absence of tumor formation induced by okadaic acid in TNF$^{-/-}$ mice. One week after initiation with 100 μg of DMBA, okadaic acid (5 μg) was applied twice a week to the skin of TNF$^{-/-}$ 129/Svj mice (●), TNF$^{+/+}$ 129/Svj mice (○), or TNF$^{+/+}$ CD-1 mice (▲). Tumors more than 1 mm in diameter were counted every week. *, $P \leq 0.01$.

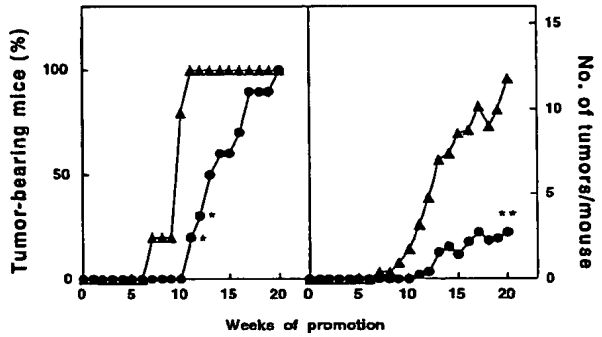

Fig. 2. Delayed tumor formation induced by TPA in TNF$^{-/-}$ mice. One week after initiation with 100 μg of DMBA, TPA (2.5 μg) was applied twice a week to the skin of TNF$^{-/-}$ mice (●) or TNF$^{+/+}$ CD-1 mice (▲). Tumors more than 1 mm in diameter were counted every week. *, $P \leq 0.01$; **, the number of tumors/mouse in TNF$^{-/-}$ mice was significantly fewer than that in TNF$^{+/+}$ CD-1 mice from 10–20 weeks ($P \leq 0.01$).

Clonal Growth of v-Ha-*ras*-transfected BALB/3T3 Cells by IL-1α and IL-1β. The transforming activity of IL-1 was studied by *in vitro* transformation of BALB/3T3 cells initiated with 3-methylcholanthrene. Although TNF-α (10.0 ng/ml) induces cell transformation *in vitro* (2), treatment with IL-1α at two concentrations (3.0 and 10.0 ng/ml) did not induce transformed foci.[5] However, in separate experiments with v-Ha-*ras*-transfected BALB/3T3 cells (Bhas 42), IL-1α and IL-1β, along with TNF-α, did induce clonal growth. Bhas 42 cells are an important model of initiated cells used to detect the tumor-promoting activity of a number of compounds (9). IL-1α and IL-1β, at various concentrations of $1–10^5$ pg/ml, dose-dependently increased the numbers of transformed foci observed in Bhas 42 cells, but not in BALB/3T3 cells without the v-Ha-*ras* gene (Fig. 3). These results suggested that IL-1 has a tumor-promoting activity that induces clonal growth in initiated cells containing a *ras* mutation but not in cells initiated by 3-methylcholanthrene but lacking the *ras* mutation.

Expression of *IL*-1α and *IL*-1β Genes in TNF$^{-/-}$ Mouse Skin Treated with Okadaic Acid and TPA. To study the role of IL-1 in tumor development in TNF$^{-/-}$ mice, we examined the expression of

[4] R. Moore, D. Owens, G. Stamp, N. East, H. Holdsworth, C. Arnott, F. Burke, M. Pasparakis, G. Kollias, and F. Balkwill reported similar results with DMBA plus TPA treatment at the 1999 meeting of the American Association for Cancer Research. Proc. Am. Assoc. Cancer Res., *40:* 98, 1999.

[5] H. Fujiki, A. Sakai, and M. Suganuma, unpublished results.

IL-1α and *IL*-1β genes in the skin. In TNF$^{-/-}$ mice, a single application of okadaic acid or TPA did not induce expression of the *TNF*-α gene. However, okadaic acid (10 μg) increased *IL*-1α and *IL*-1β gene expression about 3.1-fold and 3.7-fold, respectively, in TNF$^{-/-}$ mice 24 h after application (Fig. 4). TPA (5 μg) increased *IL*-1α and *IL*-1β gene expression about 10.8-fold and 4.5-fold in TNF$^{-/-}$ mice 4 h after application. Increased expression of *IL*-1α and *IL*-1β genes was similar to that observed in TNF$^{+/+}$ CD-1 mice (Fig. 4). It has been reported that the increases in IL-1 protein levels in mouse skin after treatment with TPA are dependent on increased *IL*-1α gene expression (14). These results suggest that residual tumor induction in TNF$^{-/-}$ mice by either TPA or okadaic acid may be dependent on IL-1 expression.

TNF-α induces transformation of BALB/3T3 cells (2), but these cells subsequently continue their growth independent of TNF-α and with elevated *IL*-1α gene expression (15). The studies with our current characterization of TNF$^{-/-}$ mice reveal that TNF-α is the instigator for transformation of cells in the early stage of tumor promotion and suggest that IL-1 also acts as TNF-α in tumor promotion. Whether okadaic acid or TPA would induce tumor promotion in mice deficient in both TNF-α and IL-1α or IL-1β remains to be investigated. Furthermore, both TNF-α and IL-1 activate various transcription factors, such as NF-κB1 and activator protein 1 (AP-1), and induce the expression of many genes. It has recently been reported that NF-κB1 and NF-κB2 are strongly overexpressed in papillomas (16); therefore, it is next of interest to study the degree of NF-κB activation in the skin tumors of TNF$^{-/-}$ mice treated with TPA or okadaic acid.

The okadaic acid class tumor promoters induce tumor promotion in three different organs (the skin, glandular stomach, and liver) initiated with three different carcinogens (7). These tumor promoters are also known to induce *TNF*-α gene expression in their target organs (17). Therefore, we believe that TNF-α is the essential molecule common to carcinogenesis in various organs and that IL-1, induced by TNF-α and okadaic acid, may be involved in the later stages of carcinogenesis, consistent with the results of an earlier study (13). Furthermore, these observations are strongly supported by the results that various cancer-preventive agents, such as green tea polyphenols, tamoxifen, and aspirin, which commonly inhibit both *TNF*-α gene expression in the cells and TNF-α release from the cells, induced by okadaic acid (18).

Although the therapeutic effects of TNF-α continue to be explored in the clinic (19), the experiments described here with TNF$^{-/-}$ mice establish a key role for TNF-α in tumor promotion. Taken together, our results suggest that TNF-α, possibly in conjunction with IL-1, plays a significant role in human carcinogenesis.

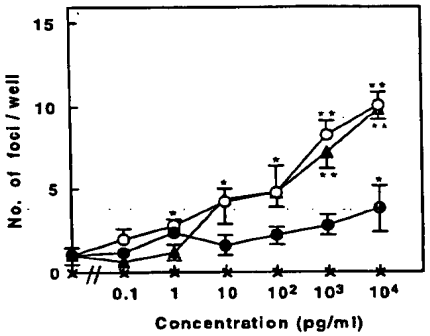

Fig. 3. Induction of clonal growth of v-Ha-*ras*-transfected BALB/3T3 cells (Bhas 42) by IL-1α, IL-1β, and TNF-α. Bhas 42 cells were treated with IL-1α (○), IL-1β (▲), and TNF-α (●) for 8 days. After treatment with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, the numbers of foci were counted. IL-1 α, IL-1β, and TNF-α (✱) did not induce any foci in BALB/3T3 cells. Values represent the means of quadruplicate analyses. *Bars*, SE; *, $P \leq 0.05$; **, $P \leq 0.01$.

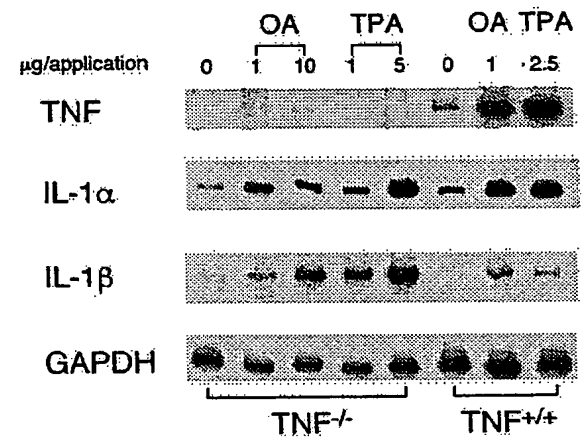

Fig. 4. Increased expression of *IL*-1α and *IL*-1β genes in the skin of TNF$^{-/-}$ mice. Total RNA was isolated from the skin of each of three TNF$^{-/-}$ mice and TNF $^{+/+}$ CD-1 mice, at 24 h after okadaic acid application (*OA*) or at 4 h after TPA application (*TPA*). The expression of *IL*-1α, *IL*-1β, and *TNF*-α genes was measured by reverse transcription-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control. The results were confirmed by two independent experiments.

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